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Association of Unstable Hemoglobin Variants and Heterozygous β-Thalassemia: Example of a New Variant Hb Acharnes or [β53(D4) Ala → Thr]

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We report here the functional and structural characterization of Hb Acharnes [β53(D4) Ala → Thr], an unstable and electrophoretically silent variant, that was found associated in *trans* with a β^0-thalassemic mutation (IVSI-1 G → A), in a patient with thalassemia intermedia syndrome. This case is discussed in comparison with other sporadic cases that we have previously investigated, resulting from the co-inheritance of a β^0-thalassemic mutation (CD39 C → T) with two other types of unstable hemoglobins, Hb Köln [β98(FG5) Val → Met], and Hb Arta [β45(CD4) Phe → Cys]. It may be concluded that, in these associated forms, both the degree of instability of the variant and the altered oxygen binding properties (affecting the degree of tissue hypoxia) are major determinants of their clinical expression. Am. J. Hematol. 62:186–192, 1999.

Key words: Hb variant; thalassemia intermedia; oxygen binding; unstable hemoglobin

INTRODUCTION

Thalassemia intermedia is an ill-defined term that refers to patients that have hematological and clinical manifestations that are milder than those observed in thalassemia major, and are most notably non-transfusion dependent. A wide variety of mutant globin gene interactions have been described to result in the clinical and hematological expression of thalassemia intermedia [1,2].

Some examples of thalassemia intermedia have been described to result from the compound heterozygosity for an unstable β chain hemoglobin (Hb) variant and a β-thalassemia mutation [3,4]. We report here a thalassemia intermedia syndrome resulting from the association of a β^0-thalassemic mutation [IVS I(−1) G → A] with Hb Acharnes [β53(D4) Ala → Thr], a new electrophoretically silent variant. Reports of β^0-thalassemia in combination with unstable Hb variants are rare, but comparison of this case, with those previously observed with Hb Köln [β98(FG5) Val → Met] [3] and Hb Arta [β45(CD4) Phe → Cys] [4] illustrates that the degree of instability and altered oxygen binding properties of the variant, (affecting the degree of tissue hypoxia), appear to play major roles in expression of the clinical severity.

MATERIALS AND METHODS

Blood samples were collected in EDTA and heparin as anticoagulants. Hemolysates were prepared by lysis of the washed erythrocytes with 4 vol of water and 0.5 vol of toluene, followed by centrifugation. Analyses were performed in Athens, Paris and Toulouse.

Hematological and Hemoglobin Studies

Hematological parameters were measured with a Technicon-Bayer H*1 whole blood autoanalyzer. Reticu-

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locytes were counted optically after methylene blue staining by an experienced hematologist.

Hemolysates were analyzed by cellulose acetate (pH 9.0), citrate agar (pH 6.2) electrophoreses and by isoelectrofocusing on agarose gel (pH 6.0–8.0). Cation-exchange high-performance liquid chromatography (CE-HPLC) was performed on hemolysates using the Bio-Rad Variant Hemoglobin testing system (Bio-Rad Labs, Hercules, CA), with either the β-thalassemia short program or the HbA_{1c} program provided by the manufacturer. Hb chains were analyzed by perfusion chromatography using a Poros R1 column [5]. Globins were analyzed by electrophoreses in urea 6 M at pH 6.0 and 9.0 and by urea-Triton polyacrylamide gel electophoresis (PAGE).

Functional Studies

Heinz body formation, thermal, and isopropyl alcohol instability tests were performed as described elsewhere [6].

Oxygen equilibrium curves (OEC) of whole blood were determined at the gas phase using the Hem-O-Scan apparatus (Aminco Travenol, Silver Spring, MA) and at the liquid phase using the Hemox-Analyzer (TCS Medical products, Southampton, PA) as previously described [7].

2,3-Diphosphoglycerate (2,3-DPG) levels were estimated enzymatically according to Ericson and de Verdier using the Boehringer kit (Mannheim, Germany) [8].

Oxygen Transport Simulation

Tissue oxygenation data were obtained either from the OECs or from Siggaard-Andersen's "Oxygen Status Algorithm" (OSA) [9], which permits computer simulations for most of data referring to oxygen delivery. Total oxygen carrying capacity parameter was calculated by multiplying the patient's haemoglobin concentration by 1.36 (quantity of O_2 bound by 1 g of fully saturated Hb and expressed as vol %). Oxygen release to the tissue at the mixed venous PO_2 parameter (oxygen extraction tension) was calculated from the OECs or/and the OSA. Cardiac output parameter (Q) was calculated by simulations using the Fick equation: $VO_2 = 0.136 \times Q \times Hb \times (SAO_2 - SVO_2)$, where: VO_2 is the amount of oxygen released per minute (l/min), Q is the blood flow (l/min), and SAO_2 and SVO_2 are the arterial and mixed venous oxygen saturations, respectively.

Erythropoietin and Soluble Transferrin Receptor Assays

Serum immunoreactive erythropoietin (Epo) levels were determined by a chemiluminescence immunoassay (Nichols Institute Diagnostics, CA). Soluble transferrin receptors level (TfR) was measured by an enzyme-linked immunosorbent assay (R&D Systems Europe, Abingdon, UK).

DNA Studies

Genomic DNA was isolated from white blood cells by the salt extraction method (10).

To localize mutations within the β globin gene, several regions of this gene were independently amplified by the polymerase chain reaction (PCR) and analyzed by denaturing gradient gel electrophoresis (DGGE). Regions of the gene displaying modified electrophoretic mobility were further analyzed by ARMS (Amplification Refractory Mutation System) PCR to identify β-thalassemia mutations common in Greece [11] and/or sequencing. The direct cycle sequencing protocol (Vistra Systems, Amersham Life Science, Amersham, UK) was performed using column-purified (Qiaquick PCR purification kit, Qiagen, USA) double-stranded PCR-generated regions of the β globin gene. The fluorescent (Texas Red) sequencing primer, was labeled using the 5′-oligonucleotide labeling kit (Vistra Systems, Amersham Life Science). The products of the sequencing reactions were analyzed using a Vistra DNA Automatic Sequencer 725 (Molecular Dynamics/Amersham).

To exclude α-globin gene rearrangements, the α globin gene cluster was mapped by Southern blot analysis using standard protocols [12]. The presence of α globin gene point mutations was investigated using DGGE analysis [13].

Characterization of the Structural Abnormality

The structural abnormality was determined as previously described [14]. The α and β chains were isolated by reversed phase HPLC and aminoethylated and the chains were digested by trypsin. The resulting peptides were separated by reversed-phase (RP) HPLC using a Vydac C_8 column (The Separation Group, Hesperia, CA). Mass spectrometry measurements were done as previously reported [15].

RESULTS

Case Report

The proband was a 6-year-old girl, anemic from early infancy (Hb ca. 90 g/l). The hematological findings included anemia, microcytosis, hypochromasia, schizocytes, basophilic stippling, reticulocytosis, and nucleated red cells. Blood chemistry showed a moderate billirubinemia. Levels of Hb A_2 and Hb F were increased. Hematological and biochemical parameters of the proband and of her parents are given in Table I. As subsequently demonstrated, the father carried the variant Hb, and the mother, a β-thalassemia mutation.

Physical examination demonstrated a subicterus and mild hepatosplenomegaly. The patient was followed up

TABLE I. Relevant Hematological, Biochemical, and Globin Gene of the Patient

	Normal range	Mother	Father	Proposita^{SPh}	Proposita^{EBV}
RBC (10^{12}/l)	4.20–6.10	5.09	5.60	4.88	4.37
Hb (g/l)	120–180	97	156	93	75
Hct (l/l)	0.37–0.52	0.34	0.47	0.31	0.27
MCV (fl)	80–99	67.8	83.0	62.7	67.7
MCH (pg)	27.0–31.0	19.0	27.8	19.0	17.1
MCHC (g/l)	325–355	305	331	303	278
RDW (%)	11.5–14.5	17.9	14.4	20.8	19.1
Retics (10^{12}/l)	0.03–0.08	0.18	0.03	0.27	0.22
Billirubin (μmol/l)	3.42–17.1	15.4	n.d.	28.9	46.2
Ferritin (μg/l)	12.0–140.0	10.0	n.d.	95.0	310.0
Epo (IU/l)	5.0–18.0	34.0	n.d.	55.8	90.0
TfR (mg/l)	0.85–3.10	7.5	n.d.	8.5	10.0
HbA_2 (%)	<3.5	5.0	3.4	6.5	6.9
HbF (%)	<1.9	1.6	0.3	2.9	2.9
α Globin genotypes		αα/αα	αα/αα	αα/αα	
β Globin genotypes[a]		$^1\beta^{0Thal}/\beta$	$\beta/^2\beta^{var}$	$\beta^{0Thal}/\beta^{var}$	

[a]1, IVSI-1 G → A; 2, cd 53 GCT → ACT or β53(D4) Ala → Thr.

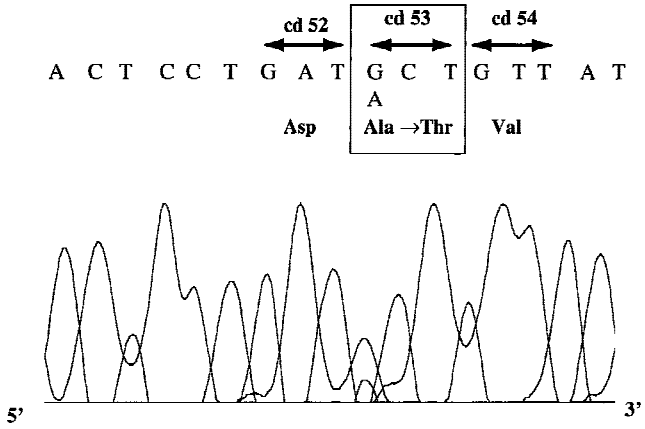

Fig. 1. DNA sequence analysis showing the GCT → ACT mutation at codon 53 of β-globin gene resulting in the aminoacid substitution Ala → Thr. The β gene on the other chromosome carried the β^0-thalassemic mutation IVS(−1) G → A (not shown).

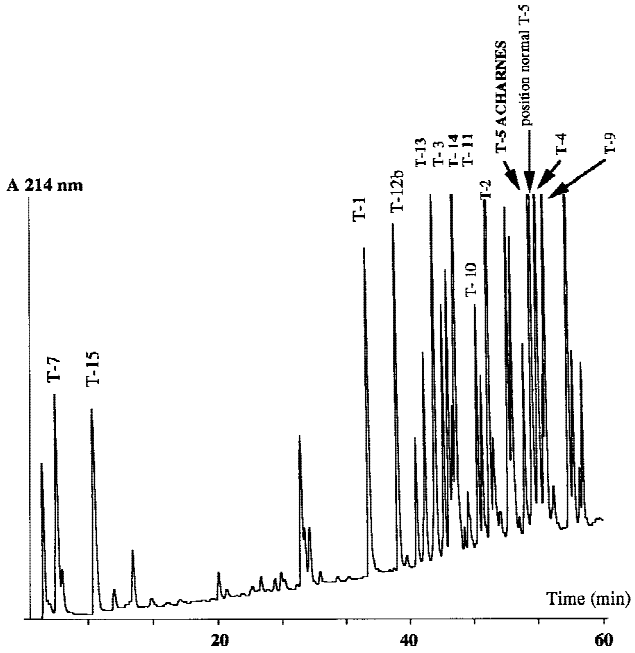

Fig. 2. HPLC pattern of the tryptic peptides of the aminoethylated β chain of Hb Acharnes showing the abnormal elution time of peptide βT-5.

in the Athens University pediatric clinic of the hospital and was hospitalized for an infectious episode due to Epstein–Barr virus (EBV). The physical examination at that time demonstrated a further enlargement of spleen and liver, and an exacerbation of the anemia (Hb 75 g/l) with a relative decrease of reticulocytosis and a more pronounced billirubinemia. The erythropoietin level of the proband was 6-fold the normal median level in our laboratory (9.2 IU/l) in the steady state condition, and this increase was more marked at the time of the EBV infection. The amount of soluble transferrin receptors was 5-fold increased compared to the normal median level in our laboratory (1.8 mg/l).

Hemoglobin Studies

No abnormal Hb component was observed by electrophoretic studies nor by CE-HPLC. No abnormal chain was found by RP-HPLC. The percentages of Hb F and Hb A_2 were 2.9% and 6.1%, respectively. This increased Hb A_2 level found in combination with a low Hb concentration was indicative of thalassemia intermedia, for which several genotypes were possible, such as the presence of extra α globin genes or of an unstable β chain Hb variant.

Characterization of Abnormal Hemoglobin

DNA studies. The proband was found to carry the β^0-thalassemic mutation IVS I(−1) G → A and a

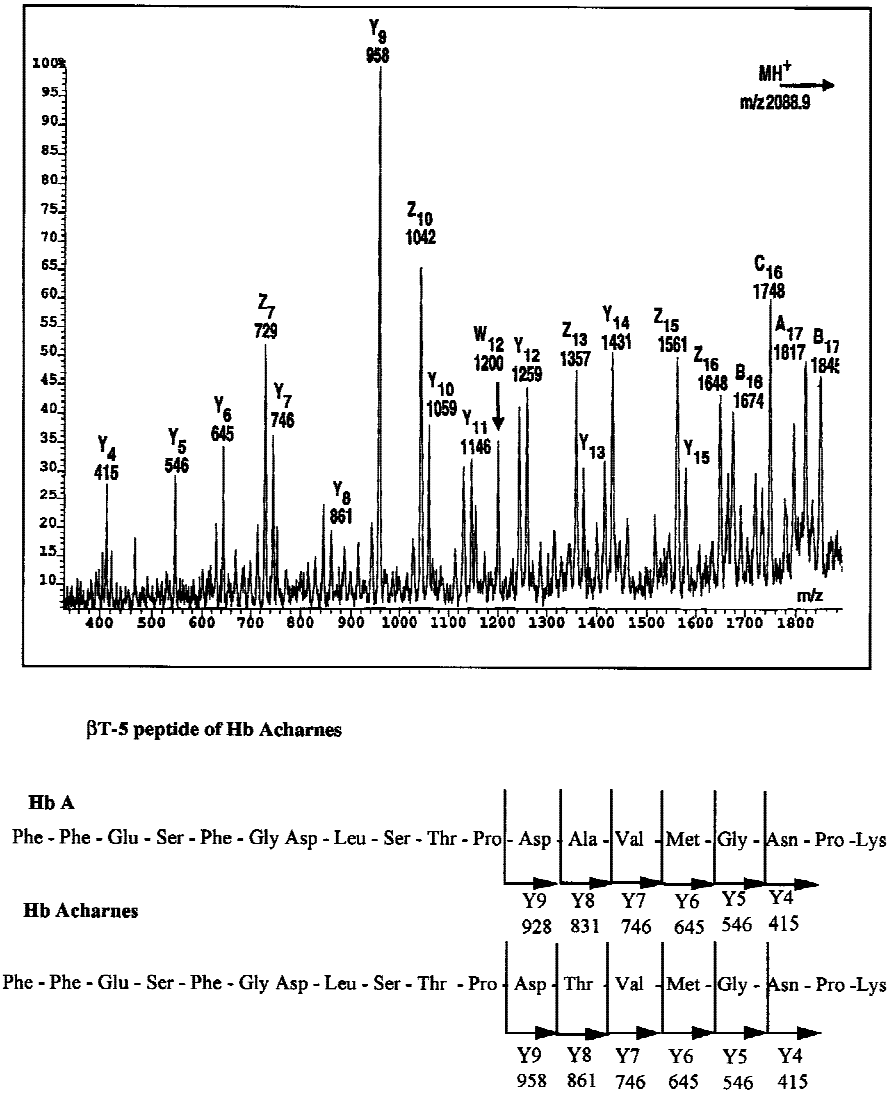

Fig. 3. Tandem mass spectrometry analysis of the abnormal βT-5 peptide. The Y series of ions shows that the 30 mu increase starts from position 7 (residue β53).

GCT → ACT mutation at codon 53 of β globin gene resulting in the aminoacid substitution Ala → Thr (Fig. 1). The presence of α-thalassemia deletions and α globin gene point mutations were excluded. Family studies showed that proband's mother carried the thalassemic mutation and her father the CD53 point mutation.

Protein studies. Electrospray analysis of the globin revealed the presence of a single signal corresponding to the β subunits but having a mass of 15897.6 Da versus 15867.3 Da for the normal.

The HPLC elution profile of the tryptic digest of the β chain revealed that the βT-5 peptide was eluted earlier than normal (Fig. 2). Tandem mass spectrometry analysis of this peptide indicated that the difference of mass concerned position 53 which is normally occupied by an alanine (Fig. 3). The only possibility for a +30 mass units shift is a replacement of this alanine by a threonine showing that the proband carried an aminoacid substitution Ala → Thr, in accordance with the GCT → ACT mutation in codon 53 found with DNA sequencing. This new Hb variant was named Hb Acharnes, from the town, an ancient municipal of the Attica district, Greece, where the patient resides.

Functional studies. Heinz body were observed after incubation of the RBC in the presence of oxidative dyes. Thermal instability was demonstrated by 50% precipitation at 65°C after 10 min incubation (vs ca. 10% for Hb A) (Fig. 4).

The OEC of fresh whole blood from the proband (which contain almost only Hb Acharnes) showed normal oxygen affinity (P_{50} = 29 mmHg) and normal cooperativity index (n_{50} = 2.5). The Hb/2,3-DPG ratio of 0.85 in the patient was within the normal range. On the basis of the OEC and the OSA, the cardiac output was calculated to be 6.2 l/min in the steady phase and 7.1 l/min during the infection (Table II).

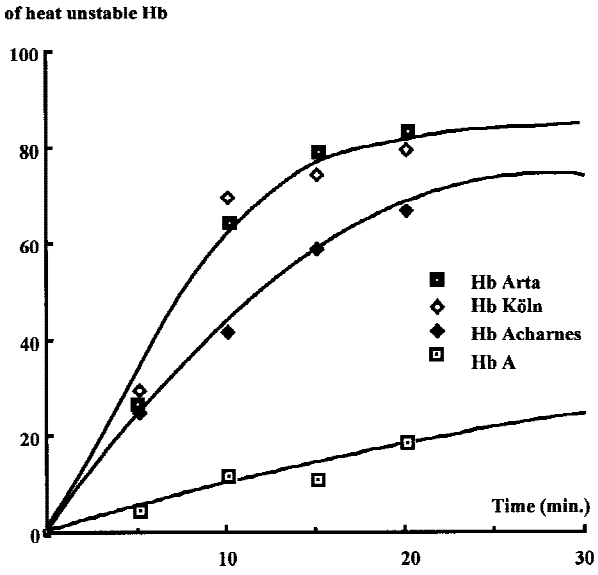

Fig. 4. Comparison of the heat unstability tests of the hemolysates of the patients carying a β^0-thalassemia in association with Hb Acharnes, Köln, or Arta.

DISCUSSION

Thalassemia intermedia usually results from the interaction of globin genotypes that cause a globin chain imbalance that is not as great as that found in thalassemia major, such as the coinheritance of an α- and β-thalassemia [2,16], heterozygous β-thalassemia with additional α globin genes [17] or β-thalassemia with coinheritance of factors causing increased γ globin chain production. Thalassemia intermedia may also be caused by hemoglobin variants, alone or on interaction with β-thalassemia, which either have reduced synthesis and/or stability [18–21]. In this latter category altered oxygen binding properties may also play a role in modifying the clinical expression. The inheritance patterns of thalassemia intermedia is quite different for those due to a highly unstable β chain, which are dominantly inherited and for those associating an allele responsible for reduced synthesis and another responsible for a moderately unstable variant.

Hb Acharnes is the first variant reported with a substitution at β53 (D4) position. This residue is external but linked to Asp β47 (CD6) and Leu β48 (CD7). Modifications affecting this latter position lead to moderately unstable variants. The structural modification of Hb Acharnes is also close to β55 Met which interacts with several residues in the neighbourhood and bridges with α119 Pro in the $\alpha_1\beta_1$ interface. The insertion of a larger and ambivalent molecule, such as threonine, in this region of the helix may affect the contacts and therefore explain the in vitro instability of this variant. As shown in Table I, this variant is clinically silent in the simple heterozygous state (in the father) but responsible for thalassemia intermedia on interaction with heterozygous β-thalassemia.

In simple β-thalassemia heterozygotes, the α subunits, which are in excess, form inclusions bodies containing aggregated α chains which behave like an unstable Hb. These inclusions are formed in the erythrocyte precursors of the bone marrow, leading to some degree of ineffective erythropoiesis. Usually, these thalassemic Heinz bodies have disappeared from the cytoplasm of the circulating erythrocytes since the protease activity is high in the erythrocyte precursors [22]. By contrast, the Heinz bodies resulting from unstable Hb variants are formed in the blood circulation under various conditions of oxidative stress. These Hb precipitates are bound by the membrane skeleton, decreasing the cell deformability and revealing abnormal epitopes favoring lysis. Ineffective erythropoiesis and hemolytic phenomenon are both observed in cases of association of β-thalassemia and unstable Hbs.

Reticulocyte production index (RPI) [23] relative to TfR levels (erythroid activity) can differentiate between ineffective erythropoiesis and peripheral hemolysis. In conditions associated with a normal proliferative response to anemia an RPI around 3 times normal with raised TfR indicates adequate red cell production (peripheral hemolysis), while an index value less than 2 times normal with raised TfR indicates impaired red cell production (ineffective erythropoiesis) [24–25]. The markedly increased TfR level in the individual with Hb Acharnes/β^0-thalassemia (10.0 mg/L), suggests a significant degree of erythron expansion comparable to that observed in other hemoglobinopathies [26]. Despite this increase in TfR levels the RPI value is relatively low (1.6), indicating that the majority of this expansion is due to ineffective erythropoiesis rather than peripheral hemolysis. Presumably the slight instability of Hb Acharnes exacerbates the effect of the excess α globin chains present because of the β^0-thalassemia carrier state.

Cases of association of heterozygous β^0-thalassemia with unstable Hb variants are exceptional. A few such cases have been reported including Hb Köln and Hb Arta leading to a thalassemia intermedia state [3,4]. As with Hb Acharnes, the stability of these two variants is reduced, in fact to a greater degree than that of Hb Acharnes (Fig. 4).

However, besides instability, oxygen binding properties of the variant may also affect the overall expression of the syndrome and is interesting to compare these three rare cases. In the quasihomozygous state, Hb Acharnes displays normal oxygen affinity and cooperativity, whereas Hb Arta has decreased oxygen affinity but normal cooperativity (n_{50} = 2.6), while Hb Köln displays high oxygen affinity and low cooperativity (n_{50} = 2.1).

TABLE II. Oxygen Transport Parameters

	Hb (g/l)	P_{50}[a] (mmHg)	Oxygen extraction pressure[b] (mmHg)	Saturation[b] (%)	Oxygen release[b] (vol%)	Cardia output[c] (l/min)
Hb Acharnes/β^0thal[d]	93	28.5	32.6	97.3	4.0	6.2
Hb Acharnes/β^0thal[e]	75	29.8	29.5	96.8	3.5	7.1
Hb Arta/β^0thal	87	37.5	39.5	95.0	5.6	4.5
Hb Köln/β^0thal	102	12.2	16.0	99.5	1.1	22.7
Normal control	150	26.1	38.0	97.4	5.3	4.7

[a]OEC measurements.
[b]Siggaard-Andersen's Oxygen status algorithm.
[c]Fick's equation.
[d]Steady phase (SPh).
[e]Infection (EBV).

The calculated oxygen transport parameters for all three variants are shown in Table II.

In Hb Acharnes the oxygen transport properties of this variant are normal, thus indicating that the reduced oxygen transport parameters and consequent increase of cardiac output are due to the degree of anemia. Conversely the decreased oxygen affinity of Hb Arta, despite its instability, resulted in normal cardiac output. It appears that the cardiac output is most affected in the case of Hb Köln due to the high oxygen affinity of the variant, despite the reduction in the oxygen extraction tension to the extremely subnormal level of 16.0 mm Hg.

Overall erythropoiesis is stimulated in increased oxygen affinity variants and decreased in low oxygen affinity variants and for this reason, under the effects of oxidative stress, the consequences, in terms of Hb level, are much more severe in the case of a low oxygen affinity unstable variant than for a high oxygen affinity unstable variant. In Hb Acharnes, since the oxygen binding properties are almost normal, the thalassemia intermedia clinical presentation is mainly the result of its instability, which, within the thalassemic erythrocytes is enhanced by the fact that the only Hb component is the abnormal one and by the presence of precipitated free α chains which may display a catalytic effect on the oxidative process [27].

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